Human Rhinovirus Type 2 Uncoating at the Plasma Membrane Is Not Affected by a pH Gradient but Is Affected by the Membrane Potential

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The minor receptor group human rhinovirus type 2 enters host cells by endocytosis via members of the low-density-lipoprotein receptor family. In late endosomes, it undergoes a conformational change solely induced by a pH of ≤5.6, resulting in RNA transfer across the endosomal membrane into the cytoplasm. To determine potential driving forces of this process, we investigated whether RNA penetration might depend on the pH gradient and/or the membrane potential between the acidic endosome lumen and the neutral cytoplasm. Since these parameters are difficult to assess in endosomes, we took advantage of the possibility of inducing structural changes, RNA release, and consequently infection from the plasma membrane. To manipulate the pH gradient, cell-bound virus was exposed to membrane-permeant or -impermeant acidic buffers at 4°C, and this was followed by a shift to 34°C in medium containing bafilomycin to prevent RNA release from endosomes. To manipulate the plasma membrane potential, similar experiments were carried out, but these included K+ diffusion potentials in the presence of the K+ ionophore valinomycin. We demonstrated that infection does not depend on a pH gradient but is enhanced by plasma membrane hyperpolarization compared to plasma membrane depolarization.

Human rhinoviruses (HRVs) are the main cause of recurrent upper respiratory tract infections known as the common cold. However, they can also affect the lower airways, leading to exacerbation of asthma and chronic obstructive pulmonary disease. HRVs belong to the family Picornaviridae, which includes important human and animal pathogens such as poliovirus, hepatitis A virus, and foot-and-mouth disease virus. Picornaviruses are nonenveloped with an icosahedral capsid composed of 60 copies of each of the four viral proteins (VP1 to VP4) that encloses a single-stranded RNA genome of about 7,100 nucleotides (35). Based on inhibition of receptor binding by a monoclonal antibody, the 99 HRVs were divided into two groups; the 87 major group viruses bind intercellular adhesion molecule 1, while the 12 minor group viruses interact with members of the low-density-lipoprotein receptor superfamily (39). HRVs enter their host cells by receptor-mediated endocytosis in clathrin-dependent and -independent manners (1, 36). On their way from early to late endosomes, they become exposed to a steadily decreasing pH controlled by the vacuolar H+-ATPase (V-ATPase) (27). Minor group viruses (exemplified by HRV type 2 [HRV2]) dissociate from the receptors at the mildly acidic pH of 6.0 in early endosomes and are delivered to endosomal carrier vesicles that maintain a pH of ≤5.6 (3). As extensively demonstrated for HRV2, below this pH threshold, the capsid undergoes structural modifications. This ultimately leads to the formation of a pore in the endosomal membrane. The process does not affect the integrity, number, or pH of the endosomes during uncoating and RNA translocation, indicating that the pores must be small and most probably open only transiently (5). Upon arrival in the cytoplasm, the RNA is translated into a polyprotein and autocatalytically cleaved into structural and nonstructural proteins; it is then replicated via a negative-strand intermediate.HRV14, which rather disrupts the endosome similarly to adenoviruses (34).

Although RNA release and penetration into the cytosol are critical steps in picornavirus infection, so far, little about what provides the driving force for membrane translocation of the viral genome is known. During entry, the virus passes through endosomal subcompartments that have been characterized mainly with respect to the pH and chloride concentration of their internal milieu (27, 37). However, membrane potential and ionic composition of their lumen are mostly unknown. The vacuolar proton pump is electrogenic, resulting in the buildup of an interior positive membrane potential. This can be demonstrated in vitro with isolated endosomes by using potential-sensitive dyes upon replacement of the permeant chloride ions with impermeant anions such as sulfate or gluconate or by collapsing the membrane potential with valinomycin (12, 16). In vivo, individual endosomes most likely maintain distinct membrane potentials as a consequence of the activity of the V-ATPase, their internal pH, chloride concentration, and other ion permeabilities and channels (15, 37). Whether the proton gradient and/or membrane potential also plays a role in viral RNA translocation has not been addressed so far.

Irurzun and Carrasco (23) studied the influence of the combination of the K+ ionophore valinomycin and the V-ATPase inhibitor concanamycin A on poliovirus entry. They observed an inhibition of productive entry and consequently of infection and hypothesized that the chemiosmotic gradient, composed

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of the membrane potential and the pH gradient, might provide the energy required for poliovirus membrane translocation. The poliovirus receptor triggers conformational changes leading to RNA release in a strictly temperature-dependent but low-pH-independent manner and RNA release occurs from ill-defined endosomes close to the plasma membrane (7). As Irurzun and Carrasco (23) demonstrated that plasma membrane hyperpolarization as well as depolarization inhibited vius entry, it is not clear which role the endosomal membrane potential plays in poliovirus RNA penetration. This prompted us to address the question in the context of RNA penetration of HRV2. We manipulated the pH gradient and the membrane potential by the use of permeant and impermeant acidic buffers and by generating K+/H+ diffusion potentials in the presence of valinomycin, respectively, and induced infection from the plasma membrane by low-pH treatment at 4°C. Conformational changes occurring in endosomes were prevented with the V-ATPase inhibitor bafilomycin, and virus replication was observed at 34°C (3). We here show that infection occurs independent of a pH gradient but is affected by the membrane potential.

MATERIALS AND METHODS

Chemicals. All chemicals were obtained from Sigma (St. Louis, MO) unless stated otherwise. Bafilomycin A1 (Alexis Corp., Lausen, Switzerland) was dissolved in dimethyl sulfoxide at 4 μM and 40 μM and stored at −20°C. BCECF-AM (2,7′-bis(2-carboxyethyl)-5(6)-carboxyfluorescein tetrakis (acetoxyethyl) ester) was purchased from Biochemica Fluka (Buchs, Switzerland), dissolved in dimethyl sulfoxide at 10 mM, and stored at −20°C. Nigericin sodium salt and valinomycin were dissolved in absolute ethanol at 10 mM and stored at 4°C. Tissue culture plates and flasks were from Ibaki (Bibby Sterilin, Stone, Staffordshire, United Kingdom). Alexa Fluor secondary antibodies were obtained from Molecular Probes (Eugene, Oregon). Bovine serum albumin (1%; PAN Biotech, Aidenbach, Germany) was dialyzed against distilled water and stored at 4°C.

Cell culture and virus propagation. HeLa-H1 cells (American Type Culture Collection [ATCC], Manassas, VA) were grown in monolayers in minimal essential tissue culture medium (MEM) supplemented with 10% heat-inactivated fetal calf serum (FCS), L-glutamine (2 mM), penicillin (100 U/ml), and streptomycin (100 μg/ml) (all from Gibco Invitrogen Corp., Paisley, United Kingdom) at 37°C and 5% CO2. For immunofluorescence microscopy, cells were seeded on 13-mm glass coverslips (Menzel, Braunschweig, Germany) or in 24-well plates for determination of the 50% tissue culture infecting doses (TCID50). Cells were seeded at a density to achieve 80% confluence after they were grown to bare coverslips.

Buffer solutions. The following buffers were used: PBS (pH 7.4) containing 125 mM NaCl, 5 mM KCl, 1 mM MgCl2, and 1 mM CaCl2 in 20 mM Na-acetate adjusted to pH 5.3 with acetic acid. EPPS buffer (pH 5.3) (71 mM K2HPO4, 20 mM NaCl, 1 mM MgCl2, and 1 mM CaCl2 in 20 mM 2-(2-hydroxyethyl)-1-piperazinopropanesulfonic acid (EPPS; Biochemica Fluka, Buchs, Switzerland) adjusted to pH 5.3 with HCl), HEPES buffer (pH 7.4) (125 mM NaCl, 5 mM KCl, 1 mM MgCl2, and 1 mM CaCl2 in 20 mM 4-(2-hydroxyethyl)-1-piperazinopropanesulfonic acid (EPPS; Biochemica Fluka, Buchs, Switzerland) adjusted to pH 5.3 with HCl), HEPES buffer (pH 7.4) (125 mM NaCl, 5 mM KCl, 1 mM MgCl2, and 1 mM CaCl2 in 20 mM HEPES adjusted with NaOH to pH 7.4), MES buffers (pH 6.0 and 6.5) (125 mM NaCl, 5 mM KCl, 1 mM MgCl2, and 1 mM CaCl2 in 20 mM 2-(N-morpholino)ethanesulfonic acid (MES) adjusted with NaOH to the respective pH), high-K+/low-H+ buffer (pH 5.3) (71 mM K2HPO4, 20 mM NaCl, 1 mM CaCl2, 1 mM MgSO4, and 10 μM valinomycin in 20 mM EPPS adjusted to pH 5.3 with HCl), and low-K+/high-H+ buffer (pH 5.3) (145 mM NaCl, 5.4 mM KCl, 1 mM CaCl2, and 1 mM MgSO4 with or without 10 μM valinomycin in 20 mM EPPS, adjusted to pH 5.3 with HCl). For the tetra[1H]phenylphosphonium bromide ([1H]TPP–) uptake assay (24), K+ buffers were supplemented with 0.2% dialyzed bovine serum albumin.

Determination of the cytoplasmic pH. HeLa cells were grown to 100% confluence on cover glass (No. 1 Coverglass for Chamber Slide; Nuc Inc., Naperville, IL) cut into 25- by 15-mm pieces. Cells were washed for 5 min in PBS and incubated with 10 μM BCECF-AM in 3 ml HEPES buffer (pH 7.4) for 40 min at room temperature for equilibration of the nonfluorescent dye (50). Intracellular esterases hydrolyze BCECF-AM to BCECF, which exhibits green fluorescence. Because of its negative charge, it is retained in cells with intact membranes. Cells were then extensively washed with ice-cold PBS++ for 10 min, and the coverslips were placed diagonally into a polystyrene cuvette with four optical sides (Surstedt, Nürnberg, Germany) containing 2.5 ml of the respective cold buffer. The cuvette was placed in a Jasco spectrophotofluorometer FP-777 (Jasco Inc., Easton, MD) with the cells facing the exciting beam at a 45° angle (30). The fluorescence intensity of intracellular BCECF was determined every other 10 min by using 440- and 490-nm excitation and 535-nm emission wavelengths at 4°C. Parameters were set to a low photomultiplier tube voltage level. The intracellular pH (pHi) was derived from the ratio of fluorescence intensity measured upon excitation at 490 nm and at the isosbestic point at 440 nm. For buffer changes (from pH 7.4 to pH 5.3 and vice versa), cells on cover glasses were transferred into new cuvettes containing the respective buffer and allowed to equilibrate for 10 min prior to the next reading. Where indicated, the K+/H+ ionophore nigericin was added at 12 μM to equilibrate the pH, and extracellular pH (pHe), Calibration curves were generated by incubating the cells in HEPES buffer (pH 7.4), MES buffer (pH 6.5 and pH 6.0), and acetate buffer (pH 5.3) in the presence of nigericin (data not shown).

Determination of the plasma membrane potential. HeLa cells grown on 15-mm Thermorax coverslips (NalgeNunc International, Rochester, NY) at confluence were washed with PBS++ and incubated in MEM for 30 min at 37°C to deplete endogenous low-density lipoprotein. The cells were then cooled to 4°C, washed, and incubated in 1 ml MEM containing 30 mM MgCl2 and 2 mM t-glutamine at 4°C for 1 h. After being washed extensively, they were incubated in 1 ml low-K+ buffer (pH 5.3) with or without valinomycin and in 1 ml high-K+ buffer (pH 5.3) with valinomycin. All buffers were supplemented with 0.16 μCi [3H]TPP– (20 to 40 Ci/mmol from GE Healthcare Bio-Sciences AB, Uppsala, Sweden). After 1 h on ice, the cells were washed once with ice-cold PBS++, blotted with a paper towel to remove excess buffer, and transferred to scintillation vials for counting. Data were corrected for nonspecific binding of [1H]TPP– to bare coverslips.

Manipulation of the pH gradient and infection from the plasma membrane. HeLa cells were washed, preincubated in 200 μl MEM with or without 200 μM bafilomycin for 30 min at 37°C, cooled to 4°C, and incubated in 200 μl MEM (with or without bafilomycin) with HRV2 at 100 TCID50/cell (i.e., 10 PFU/cell) for 1 h at 4°C. Unbound virus was removed by three washes with 2 ml ice-cold PBS++ and the cells were incubated in 200 μl acetate buffer (pH 5.3) with 200 μM bafilomycin, EPPS buffer (pH 5.3) with bafilomycin or HEPES buffer (pH 7.4) for 1 h at 4°C. The cells were then washed with ice-cold PBS++, transferred into 200 μl infection medium at 34°C, and incubated for 15 h to allow de novo protein synthesis. For viral titer determination, all samples were adjusted to the same drug concentrations. Cells and supernatant media were subjected to three freeze-thaw cycles, cell debris was removed by centrifugation, and aliquots of the supernatants were used for TCID50 determination.

Manipulation of the membrane and virus infection from the plasma membrane. HeLa cells were treated as described above for preincubation and virus binding; they were then incubated in 200 μl high-K+ buffer (pH 5.3) with valinomycin, low-K+ buffer (pH 5.3) with or without valinomycin, or HEPES buffer (pH 7.4) for 1 h at 4°C. Where indicated, 20 μM bafilomycin was added after 30 min. The percentage of HRV2 that had uncoated was calculated from the difference between the TCID50 of the virus bound to the cells at pH 7.4 and the TCID50 after low-pH treatment. In addition, to determine viral replication, the cells were washed with PBS++ transferred into infection medium with or without 200 μM bafilomycin, and incubated for 17 h at 34°C.

Indirect immunofluorescence microscopy. Cells grown on coverslips were washed with 2 ml of ice-cold PBS++ for 5 min, fixed for 30 min with 300 μl of 4% paraformaldehyde in PBS++, and quenched with 300 μl of 50 mM NaCl in PBS for 10 min. The cells were then washed (three times) and permeabilized with 300 μl of 0.2% Triton X-100 in PBS for 5 min. After the cells were washed (three times), nonspecific binding sites were blocked with 200 μl 10% goat serum (Gibco Invitrogen Corp., Paisley, United Kingdom) in PBS for 30 min. HRV2 was detected with monoclonal antibody 8F5 (10 μl) and Alexa 488-conjugated goat anti-mouse immunoglobulin G (used at 1:1,000 for 45 min at room tem-
perature; Molecular Probes Inc., Eugene, OR). Unbound antibody was removed by two washes with 5 ml PBS (10 min each). Nuclei were stained with Hoechst dye, and the cells were washed again four times for 10 min each with 5 ml PBS, briefly dipped in distilled water, and finally mounted in Mowiol. Cells were viewed with an Axiosplan 2 microscope (Carl Zeiss, Jena, Germany). Images were acquired with a multiband filter set from Carl Zeiss (excitation filter F84-490 for fluorescein isothiocyanate and the polychromatic filter F83-100 for Hoechst, fluorescein isothiocyanate, and Alexa 568 with triple-band emission filter F83-101). All images were taken at the same exposure times and processed identically by using Zeiss Axiovision software. By use of a representative mock-infected sample, a threshold was set for the fluorescence intensity of viral protein detected by indirect immunostaining.

Counting infected cells. At least 50,000 cells for each treatment were analyzed for viral infection both visually and by using the cell analysis software TissueQuest (TissueGnostics, Vienna, Austria). Briefly, monochrome images of nuclei and HRV2-specific protein staining were used to identify individual cells and to count virus-synthesizing cells, respectively. Threshold values taken from mock-infected controls were used to exclude cells not synthesizing virus from the quantification.

Determination of cellular protein synthesis. Confluent HeLa cells in 12-well plates were washed with PBS++ and incubated in 300 μl DMEM – Met/Cys for 30 min at 34°C. The plates were put on ice, and the cells were washed and incubated in 300 μl DMEM – Met/Cys containing 30 μM MgCl₂ for 1 h at 4°C. After being washed extensively, they were incubated in 300 μl high-K⁺ buffer (pH 5.3) with or without valinomycin, and HEPES buffer (pH 7.4) for 1 h at 4°C. Where indicated, 20 mM bafilomycin was added after 30 min. The cells were again washed with ice-cold PBS++ and incubated in 300 μl DMEM – Met/Cys supplemented with 2% dialyzed FCS for 3 h at 34°C. The medium was replaced with DMEM – Met/Cys containing 2% dialyzed FCS and 1 μCi/ml [35S]methionine-cysteine (20 μCi/200 μl) with or without 20 mM bafilomycin, and incubation continued for 12 h at 34°C. The cells were washed three times with ice-cold PBS++ and incubated in 300 μl EDTA-PBS (pH 7.4), harvested in 2× 300 μl PBS, pelleted at 200 × g, and resuspended in 500 μl ice-cold DMEM – Met/Cys supplemented with 2% dialyzed FCS. Ice-cold trichloroacetic acid (TCA) was added to a final concentration of 10%, and proteins were precipitated for 20 min at 4°C. After centrifugation at 17,000 × g and 4°C, 275 μl of the supernatant (TCA-soluble radioactivity) and the pellet together with the remaining supernatant (TCA-precipitable radioactivity plus TCA-soluble radioactivity) were saved. Both samples were neutralized with NaOH, and radioactivity was measured by liquid scintillation counting. The data are presented as precipitable radioactivities.

RESULTS

We have previously shown that HRV2 uncoating (including the transition to subviral particles, loss of VP4, and RNA release) and RNA translocation can be triggered at the plasma membrane by incubation of cell-bound virus with low-pH buffer at 4°C (3). Here, we made use of this setup to study the influences of pH gradient and membrane potential on RNA plasma membrane translocation. Following virus binding, the cells were manipulated in order to change their pHₜ and/or the membrane potential, uncoating was triggered, and the efficiency of RNA penetration was derived from infectivity assays.

Manipulation of the pHₜ. We chose potential membrane-permeant (acetic acid) and -impermeant (EPPS) acids for the preparation of pH 5.3 buffers to be used for changing or maintaining the pHₜ. First, it was verified that acetate buffer (pH 5.3) equilibrates the pHₜ with the pHₒ (pH equilibrium conditions) and that EPPS buffer (pH 5.3) maintains the pH gradient under conditions that lead to conformational modifications of HRV2. The pHₜ was determined with BCECF, a pH-sensitive dye. BCECF-AM, the intrinsically nonfluorescent derivative, is hydrolyzed by cytoplasmic esterases to BCECF upon cell entry. The latter is retained in living cells due to its negative charge and exhibits pH-dependent fluorescence when excited at 490 nm and pH-independent fluorescence when excited at 440 nm (30).

Cells were loaded with BCECF-AM at room temperature and cooled to 4°C, and background fluorescence was read at pH 7.4. Upon transfer of the cells into acetate buffer (pH 5.3)
and EPPS buffer (pH 5.3), the pH, was determined every other 10 min. As seen in Fig. 1, the pH, rapidly equilibrated with the pH, upon incubation in acetic buffer (pH 5.3), whereas EPPS buffer (pH 5.3) did not affect the cytosolic pH, for 60 min. The addition of the K⁺/H⁺ ionophore nigericin resulted in efficient equilibration of the pH, and pH,. Finally, cells were returned into HEPES buffer (pH 7.4) in the presence of the drug. This led to reneutralization of pH, within 20 min. Therefore, the transmembrane pH gradient can be easily dissipated with the membrane-permeable acetate buffer (pH 5.3), whereas in EPPS buffer (pH 5.3), the pH remained unchanged (i.e., the pH gradient remained undisturbed).

The pH gradient does not affect HRV2 uncoating and RNA translocation. For HRV2, the low endosomal pH per se is sufficient to trigger the conformational modification of the viral capsid; nevertheless, a pH gradient between the endosome interior and cytoplasm may facilitate uncoating and/or RNA membrane translocation. We therefore studied infection under conditions that dissipate or maintain the transmembrane pH gradient at the plasma membrane: HRV2 was bound to HeLa cells at 4°C, and the transition to subviral particles was triggered via incubation in acetic buffer (pH 5.3) and EPPS buffer (pH 5.3), respectively. The cells were then incubated at 34°C for 15 h to allow virus replication (Fig. 2A). Bafilomycin was present throughout to prevent uncoating and penetration of RNA from endosomes. Infection was assessed by the determination of (i) the percentage of cells synthesizing viral protein via indirect immunofluorescence microscopy and (ii) infectious virus produced (TCID₅₀). To exclude any influence of the buffer on the conformational changes per se, the amount of residual infectious virus was determined at time zero after incubation at pH 5.3, the levels of uncoating of the cell-bound virus was reduced to about 30% compared to infection via the natural entry route (by using HEPES buffer [pH 7.4] in the absence of bafilomycin). As evidenced from the residual infectivity at time zero after incubation at pH 5.3, the levels of uncoating (as measured via reduction of the viral titer) by the two acidic buffers were identical (Fig. 2C). Finally, no significant influence of these buffers on production of infectious virus was observed (Fig. 2D). Taken together, these experiments demonstrate that viral uncoating and RNA translocation occur to the same extent regardless of whether the transmembrane pH gradient is dissipated (pHᵢ₀ = pHᵢ) or maintained (pHᵢᵢᵢ < pHᵢ). By the same token, viral synthesis was also not significantly affected, indicating that RNA transfer through the plasma membrane into the cytosol occurred independently of the pH gradient.

Manipulation of the plasma membrane potential. The resting plasma membrane potential of HeLa cells is in the range of −52 to −77 mV (9). However, no reliable methods to measure the endosomal membrane potential in distinct subpopulations are available; presumably, the electrogenic V- H⁺-ATPase builds up an interior positive membrane potential that is not completely dissipated by the influx of chloride or efflux of cations. To investigate whether the membrane potential had any influence on HRV2 uncoating/RNA penetration, we again used the plasma membrane to mimic the situation in the endosome. The membrane potential was manipulated via potassium diffusion potentials in the presence of the K⁺ ionophore valinomycin (10). For a first step, we wanted to ascertain that depolarization and hyperpolarization can also be achieved at a low pH and at 4°C by using low- and high-K⁺ buffers (2). To this end, the level of cellular [³H]TPP⁺ uptake was determined. This lipophilic cation passively equilibrates in lipid bilayers as a function of the membrane potential (20, 24). HeLa cells were subjected to the incubation protocol summarized in Fig. 3A. At an hour prior to determination of [³H]TPP⁺ uptake, the cells were transferred into low- and high-K⁺ buffers containing [³H]TPP⁺ (see Materials and Methods), thereby establishing the conditions shown in Fig. 3B.

Effect of the membrane potential on cellular protein synthesis. To determine whether cellular protein synthesis might be affected by these treatments, control experiments were carried out. Cells were subjected to incubations similar to those illustrated in Fig. 3, and the incorporation of [³S]cysteine-methionine into cellular proteins was measured as TCA-precipitable radioactivity. In the presence of valinomycin, protein synthesis was slightly but significantly decreased compared to that seen in the absence of the drug. However, radiolabel was incorporated to about the same extent regardless of the K⁺ concentration when valinomycin was present (Fig. 4). Thus, valinomycin slightly inhibits cellular protein synthesis, and only results obtained in the presence of the drug can be compared.

Effect of the membrane potential on HRV2 uncoating and RNA translocation. HRV2 was bound to the plasma membrane, and uncoating was induced via incubation in low- and high-K⁺ buffers (pH 5.3) plus valinomycin (Fig. 5A); based on the findings described above, only samples with valinomycin were assayed. Bafilomycin was added, and the cells were shifted to 34°C to allow viral protein synthesis. After 15 h, cells synthesizing viral proteins, as detected by indirect immunofluorescence microscopy, were counted and related to the total number of cells. When uncoating of the cell-bound virus was induced via incubation in low-K⁺ buffer (pH 5.3), 15.3% of cells synthesized viral proteins, compared to only 8.5% upon incubation in high-K⁺ buffer (pH 5.3). This indicates that plasma membrane hyperpolarization stimulates uncoating/ RNA translocation (Fig. 5B). To directly analyze the transition
of native virus to subviral particles, the fraction of virus that had undergone uncoating was derived from infectious virus present prior to and after the incubations with the buffers at 4°C (Fig. 5A). As depicted in Fig. 5C, the losses of infectivity (i.e., conversion of native virus) under the two conditions were equal. Finally, we determined the viral yield after 15 h. In agreement with the results depicted in Fig. 5B, the yield of infectious virus was roughly doubled (Fig. 5D) when the cells were incubated (in the presence of valinomycin) in low-K⁺ buffer (pH 5.3) compared to high-K⁺ buffer (pH 5.3). Taken together, these data suggest that membrane translocation of the RNA is facilitated by plasma membrane hyperpolarization compared to depolarization.

**DISCUSSION**

Little is known on the driving forces for RNA translocation from the endosome interior into the cytoplasm. The transmembrane pH gradient and/or the membrane potential may
affect this process. To study the roles of the pH gradient and the membrane potential in HRV2 infection, we used the plasma membrane as a model for endosomal uncoating and RNA translocation; structural modifications of membrane-bound HRV2 were triggered by exposure to low-pH buffers (pH 5.3). Concomitantly, dependent on the composition of the respective buffer (i) the pH gradient was either dissipated or maintained between the extracellular milieu and the cytoplasm and (ii) the plasma membrane was either depolarized or hyperpolarized. The pH gradient and membrane potential were verified via BCECF and steady-state cell association of [3H]TPP, respectively. Virus uncoating and productive RNA translocation into the cytosol were quantified via the number of cells synthesizing viral proteins (by indirect immunofluorescence microscopy) and by determination of infectious virions, respectively. We showed that HRV2 infection was not affected by the pH gradient but by the plasma membrane potential.

All experiments were carried out by using the setups shown in Fig. 2A and 5A. These allow conformational modification of HRV2 via incubation in low-pH buffer at 4°C, conditions at which endocytosis does not occur. The transmembrane pH gradient could be maintained or dissipated, and the membrane potential could be manipulated even at 4°C. The critical step in the infection cycle is RNA translocation across the plasma membrane.
membrane. Given that the conformational modification per se is not affected by the low-pH buffer used, it can be assumed that manipulating the pH gradient or the membrane potential directly affects RNA transfer into the cytoplasm. The direct quantification of the amount of viral RNA arriving in the cytoplasm is currently not possible, but we have previously demonstrated a direct correlation between the amount of virus converted by the low pH and infectious virus produced (22) or the number of cells infected (4, 6). We have also successfully quantified the conversion of native HRV2 via the decrease in the viral titer (36). Together with the appropriate controls, determination of the viral titer as a function of time is thus a legitimate way of measuring uncoating followed by productive infection. It is of note that the use of infectivity assays is also the only way to exclude the possibility that the observed effects originate from the large proportion of noninfectious particles naturally present in HRV preparations rather than from those that are productive.

Another indirect way of determining the amount of viral RNA in the cytoplasm is via its translation into the polyprotein giving rise to 2A proteinase, which cleaves the initiation factor eukaryotic initiation factor 4G; degradation of eukaryotic initiation factor 4G was previously assessed in work on poliovirus (23). We also tested this method in the present study but obtained inconclusive results.

As endosomal uncoating and RNA translocation of HRV2 can be completely inhibited by bafilomycin without affecting cellular or viral protein synthesis (28, 31), RNA translocation is directly correlated with de novo viral protein synthesis and production of infectious virus, which we used here as a reliable readout. Consequently, both parameters were determined after manipulating the pH gradient or the plasma membrane potential and shifting the cells to 34°C in the presence of bafilomycin.

Role of a pH gradient in RNA translocation. In experiments by Neubauer and colleagues (28), HRV2 was internalized into HeLa cells in the presence of monensin, i.e., where uncoating is prevented, and the cells were passively acidified by incubation in acetate buffer. Under these conditions, infection was restored. This protocol leads to acidification of both the cytosol and the endosomal lumen to the same pH. This already suggested that a pH gradient was not required for RNA transfer. We now found that both the levels of virus uncoating at 4°C (as deduced from loss of infectivity) (Fig. 2C) and the virus titers attained after 15 h (Fig. 2D) were about the same regardless of the pH gradient being maintained (pHo of 5.3, pHi of 7.4) or equilibrated (pH gradient). Consequently, a pH gradient is indeed dispensable for RNA translocation. It is remarkable that infection from without (i.e., triggered by exposure to low pH at the plasma membrane) is not much less than 30% as effective as infection via the natural route despite the relatively low multiplicity of infection of 10 and virus being prebound at 4°C instead of being internalized continuously.

Potential role of the membrane potential in RNA translocation. Recently, the membrane potential of phagosomes labeled via uptake of red blood cells (5-min pulse followed by 15-min chase) was determined to be 27 mV (lumen positive) in a murine macrophage cell line (38). However, little about the in vivo membrane potential of endosomes is known, and even less about that of distinct subcompartments is known. Several
lines of evidence indicate that endosomes build up an inside positive (cytoplasmic side negative) membrane potential (11, 16, 37); the situation can thus be mimicked by the plasma membrane with its negative resting potential. To test whether uncoating and/or RNA translocation might be affected by the membrane potential, we generated potassium diffusion potentials at the plasma membrane in the presence of the K⁺/H⁺ ionophore valinomycin. The physiological membrane potential is maintained by the low K⁺/high Na⁺ concentration in the extracellular fluid. The addition of valinomycin results in hyperpolarization. In contrast, in high-K⁺/low-Na⁺ buffer valinomycin leads to depolarization. In control experiments, we verified that the same holds true for incubation at 4°C in the corresponding salt solutions buffered to pH 5.3.

![Diagram](https://via.placeholder.com/150)

**FIG. 5.** The membrane potential affects HRV2 uncoating and RNA translocation. (A) HRV2 bound to the plasma membrane at 4°C was exposed to high-K⁺ buffer (pH 5.3) or low-K⁺ buffer (pH 5.3) in the presence of valinomycin. Bafilomycin was added and then present throughout. At time zero (T = 0), cells were shifted into infection medium and incubated for 15 h at 34°C. (B) The percentage of cells producing virus was identified via immunofluorescence microscopy and related to the total number of cells stained with Hoechst dye. The means ± standard errors of the means of four independent experiments, each carried out in duplicate, are shown. The data reveal a statistically significant difference (P = 0.05; asterisk). (C) The viral titer was determined prior to (T = −60) and after (T = 0) treatment with high- and low-K⁺ buffers (pH 5.3), and the amount of virus that had uncoated was calculated from the difference between cell-bound infectious virus after incubation at pH 7.4 and that remaining after incubation at pH 5.3. (D) Infectious virus produced at 15 h after shifting to 34°C. The means ± standard errors of the means from two independent experiments, each carried out in triplicate, are shown. The difference was significant at a P value of ≤0.05 (asterisk).
mM (29) and resting potentials of $-60$ mV (low K$^+$/high Na$^+$) and 0 mV in high-K$^+$ buffer in the presence of valinomycin (24). As we found that the presence of valinomycin for 1 h at 4°C alone reduced subsequent cellular protein synthesis, only the results of experiments with valinomycin were compared. Again measuring uncoating via determination of residual infectious virus, we found no difference for hyperpolarization (Fig. 5C). However, the viral yield was increased upon hyperpolarization, demonstrating that RNA transfer into the cytosol was stimulated by a transmembrane negative potential (Fig. 5B and D).

How the endosomal membrane potential affects endosomal processes. Early on, it was observed that incubation in low-Na$^+$/high-K$^+$ medium known to lead to plasma membrane depolarization inhibited fusion and uncoating of Semliki Forest virus (SFV) (17). However, SFV enters by endocytosis, and fusion of its envelope with the membrane of early endosomes is triggered by the low luminal pH (33, 40). Thus, the mechanism of viral inhibition was not clear. More recently, voltage clamp experiments demonstrated that low-pH-induced fusion of cells expressing the SFV fusion protein E1 with the plasma membrane of target cells required transmembrane negative potentials (26). In contrast, endosomal fusion of hepatitis B virus is low-pH independent, but penetration of its DNA from endosomes into the cytosol is facilitated by the membrane potential (14).

In addition to virus fusion/uncoating, translocation of proteins across membranes is also influenced by the membrane potential. Transfer of a complex between cell-penetrating peptide 1 and beta-galactosidase is promoted by the transmembrane negative potential at the plasma membrane (18). Intoxication by bacterial toxins such as tetanus and diphtheria toxins depends on low endosomal pH and a transmembrane negative potential (21). By use of a biochemical assay for arrival in the cytoplasm, fibroblast growth factor-1 was transferred by a pH-independent but membrane potential-dependent (cytoplasmic surface negative) mechanism across intracellular vesicles, most likely recycling endosomes (25). Taken together, all these data suggest that endosomes maintain an inside positive membrane potential (10 to 20 mV), i.e., cytoplasmic side negative, due to the activity of the V-ATPase, electrogenic transporters (e.g., Na$^+$/K$^+$-ATPase, chloride/proton exchangers), and voltage-gated (e.g., sodium) channels (8, 13, 32, 37). As the latter was found in late but not early phagosomes in macrophages, this is indicative not only for distinct pH values in endosome subpopulations but also for their possessing distinct membrane potentials. In any case, fusion/genome penetration of enveloped viruses, endosomal penetration of proteins, and, as shown in the present investigation, RNA transfer of the non-enveloped virus HRV2 are stimulated by a transmembrane negative potential.

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